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Molecular Detection of Viable Bacterial Pathogens in Water by Ratiometric Pre-rRNA Analysis▽†

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Ratiometric pre-rRNA analysis (RPA) detects the replenishment of rRNA precursors that occurs rapidly upon nutritional stimulation of bacterial cells. In contrast to DNA detection by PCR, RPA distinguishes viable from inactivated bacteria. It exhibits promise as a molecular viability test for pathogens in water and other environmental samples.

As a tool for detecting bacteria in environmental samples, the PCR is limited in part by the false-positive detection of nonviable bacteria and free DNA. One solution to this problem involves treating bacteria with DNA intercalators that penetrate inactivated cells and inhibit PCR amplification (15). These methods require careful titration, and performance varies with sample and disinfection conditions (8, 16). An alternative is to detect microbial RNA, which is less stable than DNA in the environment (10–12, 14, 17–19, 23). However, mRNA can be difficult to detect, while mature rRNA is stable within inactivated cells.

Microbial rRNA precursors (pre-rRNA) comprise an alternative RNA target (2–4, 11, 17, 23). Pre-rRNAs have leader and tail sequences that are enzymatically removed during rRNA maturation. Pre-rRNA sequences are phylogenetically specific, which facilitates their detection in complex samples. In growing bacterial cells, pre-rRNAs constitute a significant fraction of the total rRNA and are much easier to detect than mRNA. Upon cessation of growth, pre-rRNA synthesis ceases but maturation continues, draining pre-rRNA pools. Pre-rRNA has been used as a steady-state indicator of bacterial physiology (2, 11); however, this strategy is compromised by the complex interplay of pre-rRNA synthesis and processing (2).

The present study exploited the replenishment of pre-rRNA that occurs immediately upon the nutritional stimulation of nutrient-limited bacterial cells. Species-specific pre-rRNA was measured in samples after brief exposure to culture medium. Values that exceeded those seen in nonstimulated control samples indicated the presence of viable cells. This ratiometric pre-rRNA analysis (RPA) approach was tested on the rapidly growing opportunistic pathogen *Aeromonas hydrophila* (generation time [g] = 1 h) and the slowly growing actinomycete *Mycobacterium avium* (g = 20 h).

For both organisms, real-time quantitative PCRs (RT-qPCRs) targeted the 5′ pre-rRNA leader region. Primers were designed to straddle the 5′ mature rRNA terminus. Primers for cDNA synthesis and reverse PCR primers recognized semiconserved sequences within the mature rRNA. Forward primers recognized predicted species-specific sequences within the 5′ leader. Therefore, amplification required intact specific pre-rRNA as templates (see Table S1 in the supplemental material).

Primers targeted to the *M. avium* complex (MAC) consistently yielded the expected amplification products when applied to 19 genotypically diverse isolates of *M. avium* subsp. *hominissuis* and *M. intracellulare* (1, 9), the two most significant human pathogens within the MAC. Nucleic acids from *M. terrae*, *M. gastri*, *M. smegmatis*, *M. nonchromogenicum*, *M. phlei*, and *M. vaccae* did not yield amplification products. Specificity of pre-rRNA primers for *A. hydrophila*, with the exclusion of the closely related fish pathogen *A. salmonicida* subsp. *salmonicida*, was predicted by BLAST analysis of the NCBI nonredundant database.

To assess the time course of pre-rRNA replenishment upon nutritional stimulation, early-stationary-phase *A. hydrophila* ATCC 7966 cells were washed, resuspended in autoclaved tap water (ATW), and incubated for 7 days with aeration at 28°C. Early-stationary-phase cells of *M. avium* subsp. *hominissuis* strain HMC02 were washed, resuspended in ATW, and then incubated for 14 days with aeration at 37°C. These conditions were designed to drain pre-rRNA pools in simulated water supply environments. To conduct RPA, bacteria in water were divided into two aliquots and centrifuged. One pellet was resuspended in culture medium (nutritional stimulation) and the other in ATW (control). Final cell densities were approximately 10^6 CFU/ml. Nutrient broth was used for nutritional stimulation of *A. hydrophila*, and Middlebrook 7H9 medium with 10% ADC supplement was used for *M. avium* subsp. *hominissuis*. After incubation for various periods of time, cells were lysed by high-energy bead beating, RNA was isolated by acidified phenol-chloroform (5, 13, 22), and pre-rRNA was measured by RT-qPCR. Pre-rRNA copy numbers were not calculated due to the lack of authentic pre-rRNA standards. However, ratios of RT-qPCR values in stimulated and control samples were calculated following normalization to genomic

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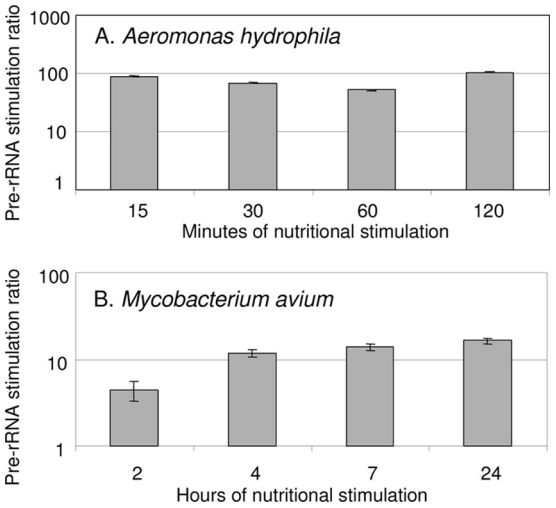

FIG. 1. Time course of nutritional stimulation of pre-rRNA in *A. hydrophila* (A) and *M. avium* strain 104 (B) cells. Pre-rRNA stimulation ratios are the ratios of pre-rRNA in stimulated samples relative to that in control samples, measured by RT-qPCR. Values are means and SDs of two or more experiments per time point. To conduct RT-qPCR on extracted RNA, cDNA was first generated using the Superscript III system (Invitrogen) and cleaned using a Qiagen PCR purification kit (catalog no. 28104). Amplification of cDNA was performed using the Applied Biosystems Power SYBR green mix. Reactions were conducted in triplicate at two different dilutions to ensure quantitative readouts. Amplifications were run in 96-well plates on an ABI Prism RT-7500 as follows: 10 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C using 9600 emulation. ABI SDS software was used to set cycle threshold values.

DNA standard curves. Pre-rRNA stimulation was very rapid in both organisms. Fifteen minutes and 4 h were adequate for near-maximum stimulation in *A. hydrophila* and *M. avium*, respectively (Fig. 1).

To assess the specificity of RPA for viable cells, sodium hypochlorite was used to generate suspensions with various ratios of viable and inactivated *A. hydrophila* cells. Briefly, *A. hydrophila* cells in water were diluted in ATW to an optical density at 600 nm of 0.1 and a 5% sodium hypochlorite reagent was added in various amounts to yield calculated initial chlorine concentrations ranging from 0 to 4 mg/liter. Suspensions were incubated at 28°C for 14 to 18 h with aeration. Percent viability (ratios of viable and inactivated cells) was determined posttreatment by plating on nutrient agar, and RPA was conducted as described earlier. In addition, DNA was quantified by standard qPCR with the same primers used for RPA. In a typical experiment (Table 1), samples with percent viabilities of 96.3%, 26.9%, and 0.02% exhibited pre-rRNA stimulation ratios of $\geq 2 \pm 1$ standard deviation (SD). Samples with no detectable viable cells (0% viability) exhibited pre-rRNA stimulation ratios of 1 or less. In contrast, qPCR detection of *A. hydrophila* genomic DNA was strongly positive in all treated and untreated samples. In four RPA experiments conducted as described for Table 1 ($n = 18$ chlorine-treated and untreated samples with various percent viabilities), pre-rRNA stimulation ratios seen in samples with no detectable CFU were significantly lower ($P = 0.0026$ by the Mann-Whitney U test) than those seen in samples with detectable CFU. No such correlation was seen when genomic DNA was quantified (Fig. 2).

To field test RPA for *A. hydrophila*, 300-ml water samples were collected from surface water sites in Seattle, WA. Three-hundred-milliliter samples were concentrated by filtration following standard protocols (6, 7). Aliquots resuspended in water were diluted twofold in 2× nutrient broth (stimulated sample) or ATW (control). After 1 h of incubation, particulates and bacteria were concentrated and RPA was conducted. Viable counts were also obtained by plating onto ampicillin-dextrin agar with vancomycin as described previously (6). Suspected *A. hydrophila* colonies were confirmed by PCR for the *ast* gene (7).

In total, three freshwater samples and one salt water sample were analyzed. The freshwater samples yielded between 280 and 798 CFU/ml *A. hydrophila*, and RPA results ranging from 4.8 ± 1.4 to 39.8 ± 12.8 (mean stimulated/control ratio). Autoclaved freshwater samples yielded no CFU and no *A. hydrophila* pre-rRNA signals. The salt water sample had 6 CFU/ml *A. hydrophila*, but no *A. hydrophila* pre-rRNA was detected. Therefore, in its current form, RPA applied to natural samples had a detection limit between 6 and 280 CFU/ml.

In practical terms, RPA can conducted by dividing a sample into two equal aliquots, one of which is nutritionally stimulated while the other is held in water or buffer. After stimulation for ≤ 1 generation time, species-specific pre-rRNA is quantified ratiometrically. The nutritional stimulation step is not of sufficient duration for even modest amplification of pathogen numbers. Thus, RPA is not a culture enrichment.

Ideally, a threshold pre-rRNA stimulation ratio of 1.0 would indicate the presence viable target cells. However, the laboratory samples in Fig. 2, which were densely populated with *A. hydrophila* cells (approximately 10^6/ml), included some samples with no detectable CFU of *A. hydrophila* and mean pre-rRNA stimulation ratios approaching 4.0. Our cultivation methods may not have been sufficiently sensitive to detect all of the viable cells in these samples. Alternatively, the samples may have contained viable but not culturable cells (20, 21) that were capable of pre-rRNA synthesis but not of colony formation. As in any detection method, threshold values for positivity are likely to be affected by sample type and pathogen load. Lower threshold values may apply to dilute natural samples.

In its present form, RPA is not quantitative. Signals that were outside of the accurate linear range of RT-qPCR, such as strongly positive stimulated samples, sometimes yielded ratios

TABLE 1. Pre-rRNA stimulation ratio and genomic DNA in suspensions of *A. hydrophila* with various ratios of viable and inactivated cells

Initial hypochlorite concn (mg/liter)	Final no. of CFU/ml	% Viability[a]	Pre-rRNA stimulation ratio[b]	No. of genomic DNA copies[c] (10^6)
0	963,000	96.3	3.0 ± 0.2	1.4 ± 0.4
1	279,000	27.9	17.2 ± 3.6	3.8 ± 0.5
2	190	0.02	73.0 ± 54.2	4.0 ± 0
3	0	0	0.6 ± 1.0	3.8 ± 0.5
4	0	0	0.04 ± 0.1	5.4 ± 0.6

[a] Normalized to estimated 1×10^6 input bacteria.
[b] Mean ± SD of three replicate samples.
[c] Extracted by phenol-chloroform method; mean ± SD of replicate two samples.

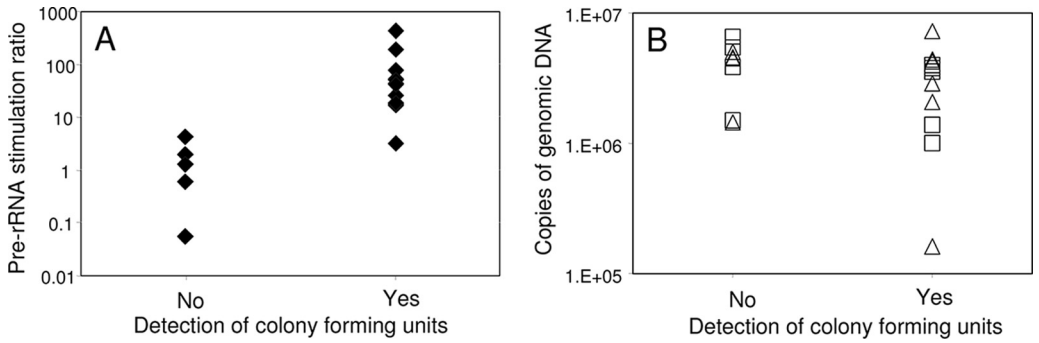

FIG. 2. Correlation between the presence of viable *A. hydrophila* cells and the pre-rRNA stimulation ratio (A) or genomic DNA quantified by qPCR (B) in chlorine-treated laboratory suspensions. Pre-rRNA stimulation ratios (A) are the ratios of pre-rRNA in stimulated samples relative to that in control samples measured by RT-qPCR. Values are means of three measurements per sample. Numbers of genomic DNA copies (B) were quantified by qPCR normalized to a genomic DNA standard curve. DNA was measured in stimulated (open squares) and nonstimulated (open triangles) samples.

that were not numerically proportionate to viable counts. Optimization and standardization for dilute samples may enable quantitative readouts.

In summary, our results support the feasibility of RPA as a means to specifically detect viable pathogens in environmental samples. The method reduced the number of false-positive results obtained with samples containing only dead bacterial cells and DNA. It should also reduce the number of false positives caused by laboratory contamination of samples or PCR reagents. RPA is robust and built upon a physiological feature of all bacteria. It may prove broadly useful in food and water safety analysis, either by itself or as an adjunct to other tools.

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